9-15-06

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TRANSMITTAL FORM

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Total Number of Pages in This Submission	19

Application Number	Not Yet Assigned
Filing Date	May 26, 1999
First Named Inventor	Michael G. Rosenblum
Art Unit	1643
Examiner Name	K. A. Canella
Attorney Docket Number	AH-CLFR:092US

ENCLOSURES (*Check all that apply*)

- [x] Fee Transmittal Form
- [ ] Fee Attached
- [ ] Amendment/Reply
- [ ] After Final
- [ ] Affidavits/declaration(s)
- [ ] Extension of Time Request
- [ ] Express Abandonment Request
- [ ] Information Disclosure Statement
- [ ] Certified Copy of Priority Document(s)
- [ ] Reply to Missing Parts/ Incomplete Application
- [ ] Reply to Missing Parts under 37 CFR 1.52 or 1.53
- [ ] Drawing(s)
- [ ] Licensing-related Papers
- [ ] Petition
- [ ] Petition to Convert to a Provisional Application
- [ ] Power of Attorney, Revocation Change of Correspondence Address
- [ ] Terminal Disclaimer
- [ ] Request for Refund
- [ ] CD, Number of CD(s) _____
- [ ] Landscape Table on CD
- [ ] After Allowance Communication to TC
- [ ] Appeal Communication to Board of Appeals and Interferences
- [x] Appeal Communication to TC **(Appeal Notice and Brief)**
- [ ] Proprietary Information
- [ ] Status Letter
- [x] Other Enclosure(s) (please Identify below):

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Remarks

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Firm Name	FULBRIGHT & JAWORSKI L.L.P.		
Signature			
Printed name	Melissa L. Sistrunk		
Date	September 14, 2006	Reg. No.	45,579

Transmittal

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being deposited with the U.S. Postal Service as Express Mail, Airbill No. EV 678183872US, on the date shown below in an envelope addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Dated: September 14, 2006 Signature: _____ (Monica T. Owens)

25696749.1

PTO/SB/17 (07-06)
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U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Effective on 12/08/2004.
Fees pursuant to the Consolidated Appropriations Act, 2005 (H.R. 4818).

FEE TRANSMITTAL
For FY 2005

[x] Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT ($) 180.00

Complete if Known	
Application Number	Not Yet Assigned
Filing Date	May 26, 1999
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Art Unit	1643
Attorney Docket No.	AH-CLFR:092US

METHOD OF PAYMENT (check all that apply)

[ ] Check [ ] Credit Card [ ] Money Order [ ] None [ ] Other (please identify): _____

[x] Deposit Account Deposit Account Number: 06-2375 Deposit Account Name: Fulbright & Jaworski L.L.P.

For the above-identified deposit account, the Director is hereby authorized to: (check all that apply)

[x] Charge fee(s) indicated below
[ ] Charge fee(s) indicated below, **except for the filing fee**
[x] Charge any additional fee(s) or underpayment of fee(s) under 37 CFR 1.16 and 1.17
[x] Credit any overpayments

FEE CALCULATION

1. BASIC FILING, SEARCH, AND EXAMINATION FEES

	FILING FEES		SEARCH FEES		EXAMINATION FEES		
Application Type	**Fee ($)**	**Small Entity Fee ($)**	**Fee ($)**	**Small Entity Fee ($)**	**Fee ($)**	**Small Entity Fee ($)**	**Fees Paid ($)**
Utility	300	150	500	250	200	100	
Design	200	100	100	50	130	65	
Plant	200	100	300	150	160	80	
Reissue	300	150	500	250	600	300	
Provisional	200	100	0	0	0	0	

2. EXCESS CLAIM FEES

Fee Description	**Fee ($)**	**Small Entity Fee ($)**
Each claim over 20 (including Reissues)	50	25
Each independent claim over 3 (including Reissues)	200	100
Multiple dependent claims	360	180

Total Claims		**Extra Claims**	**Fee ($)**		**Fee Paid ($)**
_____	- 20 =	_____	x _____	=	_____

HP = highest number of total claims paid for, if greater than 20.

Indep. Claims		**Extra Claims**	**Fee ($)**		**Fee Paid ($)**
_____	- 3 =	_____	x _____	=	_____

HP = highest number of independent claims paid for, if greater than 3.

Multiple Dependent Claims

Fee ($)	**Fee Paid ($)**
_____	_____
_____	_____

3. APPLICATION SIZE FEE

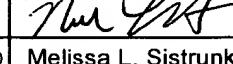
If the specification and drawings exceed 100 sheets of paper (excluding electronically filed sequence or computer listings under 37 CFR 1.52(e)), the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).

Total Sheets		**Extra Sheets**		**Number of each additional 50 or fraction thereof**		**Fee ($)**		**Fee Paid ($)**
_____	- 100 =	_____	/50	_____	(round up to a whole number) x	_____	=	_____

4. OTHER FEE(S)

	Fees Paid ($)
Non-English Specification, $130 fee (no small entity discount)	
Other (e.g., late filing surcharge): 2401 Notice of appeal / Restatement of Appeal	90.00
2402 Filing a brief in support of an appeal / Restatement Appeal Brief	90.00

SUBMITTED BY

Signature		Registration No. (Attorney/Agent)	45,579	Telephone	(713) 651-3735
Name (Print/Type)	Melissa L. Sistrunk			Date	September 14, 2006

Fee Transmittal

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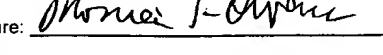
Dated: September 14, 2006 Signature:  (Monica T. Owens)

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Dated: September 14, 2006 Signature: Monica T. Owens
(Monica T. Owens)

Docket No.: AH-CLFR:092US
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Rosenblum *et al.*

Application No.: 09/320,156

Filed: May 26, 1999

Art Unit: 1643

For: IMMUNOTOXINS DIRECTED AGAINST C-ERBB2 (HER-2/NEU) RELATED SURFACE ANTIGENS

Examiner: Canella, K.A.

SECOND NOTICE OF APPEAL AND

REQUEST FOR REINSTATEMENT OF APPEAL NO. 2005-2347

MS Appeal Brief-Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sirs:

This submission concerns an appeal to the Board of Patent Appeals and Interferences from the last decision of the Examiner. The original Notice of Appeal was filed for this case on November 22, 2002, and the original Brief was filed on January 13, 2003. On August 18, 2005, an Order Remanding Appeal to Examiner was mailed, and on June 14, 2006, the Examiner issued an Office Action.

This second notice of appeal is submitted to reinstate the Appeal No. 2005-2347 or, in the alternative, if the appeal will not be reinstated this filing is a Notice of Appeal for an additional Appeal Brief, which is submitted herewith.

Applicants consider the fee for this Notice of Appeal fee and brief fee to be the difference between the fee for the original Notice of Appeal and the original Brief filed January 13, 2003 ($90) and the current fee for Notice of Appeal and filing a Brief ($90) for a total fee of $180. If

Adjustment date: 09/18/2006 RMEBRAHT
01/17/2003 JADDO1 00000094 071185 09320156
01 FC:2402 25696523.160.00 CR

Adjustment date: 09/18/2006 RMEBRAHT
12/02/2002 SLUANG1 00000014 071185 09320156
02 FC:2401 160.00 CR

09/18/2006 RMEBRAHT 00000054 062375 09320156
01 FC:2401 250.00 DA
02 FC:2402 250.00 DA

any additional fees are required, please charge the Deposit Account 06-2375 under the reference number AH-CLFR:092US, from which the undersigned is allowed to withdraw.

Dated: Sept. 14, 2006

Respectfully submitted,

By _____

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Agent of Record for Applicant

Docket No.: AH-CLFR:092US
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Rosenblum *et al.*

Application No.: 09/320,156

Filed: May 26, 1999

Art Unit: 1643

For: IMMUNOTOXINS DIRECTED AGAINST C-ERBB2 (HER-2/NEU) RELATED SURFACE ANTIGENS

Examiner: Canella, K.A.

APPEAL BRIEF

Commissioner for Patents
Washington, D.C. 20231

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TABLE OF CONTENTS

Docket No.: AH-CLFR:092US
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Rosenblum *et al.*

Application No.: 09/320,156

Filed: May 26, 1999

Art Unit: 1643

For: IMMUNOTOXINS DIRECTED AGAINST C-ERBB2 (HER-2/NEU) RELATED SURFACE ANTIGENS

Examiner: Canella, K.A.

APPEAL BRIEF

MS Appeal Brief
Commissioner of Patents
Washington, D.C. 20231

Sir:

Appellants hereby submit an Appeal Brief to the Board of Patent Appeals and Interferences.

The original Notice of Appeal was filed for this case on November 22, 2002, and the original Brief was filed on January 13, 2003. On August 18, 2005, an Order Remanding Appeal to Examiner was mailed, and on June 14, 2006, the Examiner issued an Office Action. A Second Notice of Appeal and Request for Reinstatement of Appeal is filed herewith.

Applicants consider the fee for this Notice of Appeal fee and brief fee to be the difference between the fee for the original Notice of Appeal and the original Brief filed January 13, 2003 ($90) and the current fee for Notice of Appeal and filing a Brief ($90) for a total fee of $180. If any additional fees are required, please charge the Deposit Account 06-

2375 under the reference number AH-CLFR:092US, from which the undersigned is allowed to withdraw.

Please date stamp and return the attached postcard as evidence of receipt.

I. REAL PARTY IN INTEREST

The real party in interest is the assignee, Research Development Foundation.

II. RELATED APPEALS AND INTERFERENCES

There are no related appeals or interferences.

III. STATUS OF THE CLAIMS

Claims 1-21 were originally filed. Claims 1-14 and 20-21 are withdrawn. Claim 18 was canceled. Claims 15-17 and 19 are under examination and are the subject of appeal.

IV. STATUS OF AMENDMENTS

There are no pending amendments.

V. SUMMARY OF THE CLAIMED SUBJECT MATTER

The present invention generally concerns compositions related to conjugates or fusion proteins of tumor necrosis factor (TNF) to scFv-23, a single chain antibody that exhibits binding specificity for an extracellular epitope of c-erbB-2, as represented in claims 15 and 16, respectively, and as described in the specification at least on page 5, line 13-page 6, line 2. In particular aspects the invention further concerns the fusion protein being recombinantly produced by fusing a gene encoding the single chain antibody to a gene encoding TNF, as represented in claim 17, and as described in the specification at least on page 5, line 13-page 6, line 2. In further aspects of the invention, the conjugate composition is comprised in a pharmaceutically acceptable vehicle, as represented in claim 19, and as described on page 48, lines 5-9.

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

Claims 15 and 19 are rejected under 35 U.S.C. §103(a) as allegedly being unpatentable over King *et al.* (U.S. Patent No. 5,587,458; "King") in view of Rosenblum *et al.* (Cancer Communications, 1991, Vol. 3, pp. 21-27; "Rosenblum").

Claims 15-17 and 19 are rejected under 35 U.S.C. §103(a) as allegedly being unpatentable over King and Rosenblum and further in view of Gillies (U.S. Patent No. 5,650,150).

VII. ARGUMENT

A. Substantial Evidence Required to Uphold the Examiner's Position

As an initial matter, Appellants note that findings of fact and conclusions of law by the U.S. Patent and Trademark Office must be made in accordance with the Administrative Procedure Act, 5 U.S.C. § 706(A), (E), 1994. *Dickinson v. Zurko*, 527 U.S. 150, 158 (1999). Moreover, the Federal Circuit has held that findings of fact by the Board of Patent Appeals and Interferences must be supported by "substantial evidence" within the record. *In re Gartside*, 203 F.3d 1305, 1315 (Fed. Cir. 2000). In *Gartside*, the Federal Circuit stated that "the 'substantial evidence' standard asks whether a reasonable fact finder could have arrived at the agency's decision." *Id.* at 1312.

Accordingly, it necessarily follows that an Examiner's position on Appeal must be supported by "substantial evidence" within the record in order to be upheld by the Board of Patent Appeals and Interferences.

B. Issues under 35 U.S.C. §103(a)

1. King in view of Rosenblum

Claims 15 and 19 are rejected under 35 USC §103(a) as being unpatentable over King *et al.* (U.S. Patent No. 5,587,458; "King") and Rosenblum *et al.* (Cancer Communications, 1991, vol. 3, pp. 21-27; "Rosenblum"). Appellants respectfully disagree.

There is no motivation or suggestion to combine Rosenblum and King, and therefore the Examiner has failed to make a *prima facie* case of obviousness. King concerns a conjugate of single chain anti-erbB-2 antibodies to a cytotoxic moiety, and Rosenblum describes conjugation of TNF to a murine antibody, ZME-018, which binds an epitope of a high molecular weight antigen, gp240, found on the surface of some melanoma cells. King makes no mention or suggestion to employ TNF as a cytotoxic moiety and, more importantly, provides no motivation to employ TNF. In column 8, lines 47-54, King teaches the skilled artisan to employ antibodies attached to cytotoxic moieties, such as radioactive materials, anti-cancer drugs, anti-metabolies, inhibitors of protein synthesis, and agents that bind DNA. None of these are cytokines, and there is no suggestion to use cytokines. King explicitly provides a list of usable moieties and yet excludes cytokines in the list, so the skilled artisan would have no motivation or suggestion to employ cytokines, and certainly not to utilize TNF specifically.

In fact, King also provides no motivation or suggestion to combine with another reference, and certainly not Rosenblum, because King heralds the use of conjugation of the antibody e23 to the exemplary *Pseudomonas* exotoxin A variant PE40 and derivatives thereof as cytotoxic moieties (see at least column 8, line 47-column 9, line 11 and column 18, line 60-column 21, line 19), so there would be no motivation to utilize any reagents other than what is taught by King as being successful or as alternatives thereto. Given that King reports success with the technology, one of skill in the art would find no motivation to expend time and resources to alter successful protocols.

Appellants note that the person of ordinary skill in the art is an objective legal construct presumed to think along conventional lines without undertaking to innovate, whether by systematic research or by extraordinary insights. *Life Technologies, Inc. v. Clontech Laboratories, Inc.*, 224 F.3d 1320, 56 U.S.P.Q.2d 1186 (Fed. Cir. 2000), citing *The*

Standard Oil Co. v. American Cyanamid Company, 774 F.2d 448, 227 U.S.P.Q. 293 (Fed. Cir. 1985), which states the following:

> The statutory emphasis is on a person of ordinary skill. Inventors, as a class, according to the concepts underlying the Constitution and the statutes that have created the patent system, possess something--call it what you will--which sets them apart from the workers of ordinary skill, and one should not go about determining obviousness under §103 by inquiring into what patentees (*i.e.*, inventors) would have known or would likely have done, faced with the revelations of references. **A person of ordinary skill in the art is also presumed to be one who thinks along the line of conventional wisdom in the art and is not one who undertakes to innovate,** whether by patient, and often expensive, systematic research or by extraordinary insights, it makes no difference which (emphasis added).

Even if one were motivated to combine the teachings of King with another reference, which Appellants do not acquiesce, there is no motivation or suggestion to specifically combine King with Rosenblum. Rosenblum similarly reports success with his technology (see at least right column or p. 23-right column of p. 25; "impressive growth inhibition effects" (p. 25, right column)). Also, King was filed two years after Rosenblum, and yet King chose not to describe or even suggest conjugating scFv23 to TNF even though a variety of cytotoxic moieties were listed in King (see at least column 8, lines 47-54). In addition, by teaching use of a monoclonal antibody, Rosenblum teaches away from using a single chain antibody. By describing conjugation of TNF to an antibody specific for a melanoma, Rosenblum at most might lead one of skill in the art to crosslink TNF to an alternative monoclonal antibody specific for a surface epitope of a melanoma cell. Even if Appellants were to acquiesce that Rosenblum provides motivation to utilize antibodies other than ZME-018 for delivery of TNF, which they do not, there is no guidance or suggestion in King which of the plethora of antibodies in the art to select from.

Appellants thus contend that the Examiner has engaged in an improper hindsight reconstruction, picking and choosing from King and Rosenblum that differ in their teachings.

"One cannot use hindsight reconstruction to pick and choose among isolated disclosures in the prior art to depreciate the claimed invention." *In re Fina*, 5 USPQ2d 1596, 1600 (Fed. Cir. 1988).

Appellants also respectfully remind the Examiner that section 103 requires consideration of the claimed invention "as a whole." This "as a whole" requirement prevents evaluation of the invention part by part, in hindsight. *Envtl. Designs, Ltd. v. Union Oil Co.*, 713 F.2d 693, 698 (Fed. Cir. 1983). Without this requirement, an obviousness assessment could break an invention into its component parts (in specific embodiments, *e.g.*, conjugation of scFv23 antibody and TNF), then find a prior art reference containing the component parts (*e.g.*, conjugation of scFv23 as described by King and TNF as described by Rosenblum), and on that basis alone declare the invention obvious. The courts have refused to act on this type of hindsight reasoning, which uses the invention as a roadmap to find its prior art components. This type of analysis discounts the value of novel selection inventions. Thus, the courts have required that an Examiner must show some suggestion or motivation, *excluding the invention itself*, to make the new combination. See *In re Rouffet*, 149 F.3d 1350, 1355-56 (Fed. Cir. 1998); *In re Lee* 277 F. 2d 1338, 61 USPQ 2d 1430 (Fed. Cir. 2002); and *c.f. Ruiz v. A.B. Chance Co.*, F.3d 1270 (Fed. Cir. 2004) (emphasis added).

Furthermore, on page 3 of the Action, the Examiner states that one of skill in the art would have been motivated to substitute TNF for the cytotoxic moiety employed in King. As noted in MPEP 2144.03 and in keeping with *In re Zurko* (258 F.3d 1385, 59 USPQ2d 1697 (Fed. Cir. 2001)), an assessment of basic knowledge and common sense that is not based on any evidence in the record lacks substantial evidence support, and the Examiner has provided no evidentiary support for the assertion that one of skill in the art would be motivated to change the cytotoxic moiety of King to TNF. The Examiner must provide specific factual findings predicated on sound technical and scientific reasoning to support his or her

conclusion of common knowledge. *In re Chevenard*, 139 F.2d 713, 60 USPQ 241 (CCPA 1943). *In re Soli*, 317 F. 2d 941, 945-946, 137 USPQ 797, 800 (CCPA 1963). Moreover, if the Appellant adequately traverses the Examiner's assertion of official notice, the Examiner must provide documentary evidence in the next action if the rejection is to be maintained. See 37 CFR §1.104 (c)(2) and *Zurko*, 258 F.3d at 1386, 59 USPQ2d at 1697. If the Examiner is relying on personal knowledge to support the finding of what is known in the art, the Examiner must provide an affidavit or declaration setting forth specific factual statements and explanation to support the finding. See 37 CFR §1.104(d)(2).

Official notice unsupported by documentary evidence should only be taken by the examiner where the facts asserted to be well-known, or to be common knowledge in the art, are capable of instant and unquestionable demonstration as being well-known. As noted by the court in *In re Ahlert*, 424 F.2d 1088, 1091, 165 USPQ 418, 420 (CCPA 1970), the notice of facts beyond the record which may be taken by the examiner must be "capable of such instant and **unquestionable demonstration as to defy dispute**" (citing *In re Knapp* Monarch Co., 296 F.2d 230, 132 USPQ 6 (CCPA 1961)) (emphasis added).

Appellants assert that the claims are not unpatentable over King in view of Rosenblum at least because there is no motivation or suggestion to combine the references, and Appellants respectfully request reversal of the rejection.

2. King and Rosenblum in view of Gillies

Claims 15-17 and 19 are rejected under 35 USC §103(a) as being unpatentable over King in view of Rosenblum and further in view of Gillies *et al.* (U.S. Patent No. 5,650,150; "Gillies"). Appellants respectfully disagree.

a) Claims 15 and 19

As argued in Section VII.B.1. of this Brief, claims 15 and 19 are not unpatentable over the combination of King and Rosenblum given that neither King nor Rosenblum provide

suggestion or motivation to combine with each other. It follows, then, that there can be no motivation to combine these references with any other reference, including Gillies. Furthermore, the Examiner cites Gillies as an obvious combination with King and Rosenblum because Gillies teaches a recombinant fusion of TNF-alpha to the heavy chain variable region of an antibody, yet by teaching a recombinant fusion of TNF to scFv23 Gillies teaches away from the conjugate nature of the subject matter of claims 15 and 19.

b) Claims 16 and 17

The Examiner alleges that claims 15-17 and 19, but particularly claim 16, are further obvious in view of Gillies because Gillies concerns making recombinant fusion of TNF-alpha to the heavy chain variable region of an antibody. As discussed above, the claims are not unpatentable over the combination of King and Rosenblum because there is no motivation or suggestion to combine King and Rosenblum with each other; therefore, there can be no motivation to combine these references with any other reference, including Gillies.

Appellants assert that even if there were motivation or suggestion to combine King with Rosenblum, which Appellants do not acquiesce, there is furthermore no motivation to combine King and Rosenblum with Gillies. Gillies concerns recombination of TNF with a heavy chain variable region of an antibody. Having an earlier filing date than King and Rosenblum, the skilled artisan would be aware of Gillies' techniques to employ recombinant fusions, and yet neither King nor Rosenblum chose to utilize such methods in their working Examples. Therefore, one of skill in the art would similarly not be motivated to employ recombination fusions over the preferred conjugation techniques of King and Rosenblum.

Appellants respectfully request reversal of the rejection.

VIII. CONCLUSION

Appellants have provided arguments that overcome the pending rejection. Appellants respectfully submit that the Office Action's conclusions that the claims should be rejected are unwarranted. It is therefore requested that the Board overturn the Action's rejections.

Please date stamp and return the enclosed postcard to evidence receipt of this document.

Dated: Sept. 14, 2006

Respectfully submitted,

By _____

Melissa L. Sistrunk
Registration No.: 45,579
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Agent for Applicant

APPENDIX 1

CLAIMS ON APPEAL

15. A composition comprising a conjugate of tumor necrosis factor to a single chain antibody exhibiting binding specificity for an extracellular epitope of c-erbB-2 protein, wherein said single chain antibody is scFv-23.

16. A composition comprising a fusion protein of tumor necrosis factor to a single chain antibody exhibiting binding specificity for an extracellular epitope of c-erbB-2 protein.

17. The composition of Claim 16, wherein said fusion protein is recombinantly produced by fusing a gene encoding said single chain antibody to a gene encoding said tumor necrosis factor.

19. The pharmaceutical composition, comprising the composition of claim 15 and a pharmaceutically acceptable vehicle.

APPENDIX 2

EVIDENCE APPENDIX

Exhibit 1. King *et al.* (U.S. Patent No. 5,587,458) made of record in the Office Action mailed June 14, 2006

Exhibit 2. Rosenblum *et al.* (Cancer Communications, 1991, Vol. 3, pp. 21-27) made of record in the Office Action mailed April 26, 2000

Exhibit 3. Gillies (U.S. Patent No. 5,650,150) made of record in the Office Action mailed June 14, 2006

Cancer Communications, Vol. 3, No. 1, 1991, pp. 21–27
Printed in the U.S.A.

0955-3541/91 $3.00 + 0.00

Antibody-Mediated Delivery of Tumor Necrosis Factor (TNF-α): Improvement of Cytotoxicity and Reduction of Cellular Resistance

Michael G. Rosenblum[a], Lawrence Cheung, James L. Murray, and Richard Bartholomew[b]

Immunopharmacology Section, Department of Clinical Immunology and Biological Therapy,
The University of Texas, M. D. Anderson Cancer Center,
1515 Holcombe Blvd, Box 041, Houston, TX 77030

(Submitted October 16, 1990; sent for revision October 26; received October 31; accepted November 5, 1990)

Abstract. Recombinant human tumor necrosis factor-α (TNF-α) is a macrophage-derived, non-glycosylated (17 kDa) peptide that has a remarkably broad range of biological and immunological effects including antiviral action and cytotoxic and cytostatic effects. TNF-α was coupled to murine antibody ZME-018, which recognizes a 240 kDa glycoprotein present on over 80% of melanoma cells. The crosslinking was accomplished using the heterobifunctional crosslinking reagent, *N*-succimindyl 3-(2-pyridyldithio) proprionate (SPDP). After purification on gel-permeation and affinity columns, the resulting eluate was analyzed by non-reducing SDS-PAGE, which confirmed that the product was a mixture of ZME-018 coupled to one or two TNF-α molecules. The ZME-TNF conjugate was titered against murine L-929 cells to demonstrate the presence of active TNF. ELISA of the conjugate against target BRO human melanoma cells or non-target T-24 cells demonstrated specific binding only to target cells. Melanoma BRO cells were killed by the immunoconjugate (IC_{50} of 10 units/mL), whereas native TNF-α had no effect at concentrations >50,000 units/mL. The immunoconjugate and TNF-α were inactive against T-24 non-target cells. These studies suggest that the sensitivity of cells to TNF was dramatically augmented by specific antibody mediated delivery to tumor cells.

Murine monoclonal antibodies to human tumor cell surface antigens have the unique ability to distribute to and accumulate within human tumors after systemic administration [1–5]. Numerous efforts have been made to exploit the selectivity and specificity of these reagents for cancer therapy [6–10]. Radiolabeled antibodies to human melanoma antigens [11, 12], to CEA[c] [13, 14], and to other cell-surface antigens [1, 15–19] exhibit specific tumor localization in patients after administration.

Murine antibody ZME-018 binds to epitope "a" of a high molecular weight antigen (gp240) found on the surface of more than 80% of melanoma cell lines and fresh tumor samples [20]. When labeled with ^{111}In and administered systemically to patients with melanoma, ZME-018 has been found to localize in 77% of soft tissue melanoma lesions [21].

Because of their unique abilities to localize within human tumors after systemic administration, antibodies have the potential to serve as targeting vehicles for specific delivery of cytotoxic chemotherapeutic agents, toxic peptides, biological response modifiers, and therapeutic radionuclides.

TNF-α is a polypeptide secreted primarily by activated macrophages [22], and it shares approximately 30% structural homology with another peptide hormone, LT (also termed TNF-β), that is secreted by activated lymphocytes [23]. Studies by Beutler et al. [24], Carswell et al. [25], and others [26, 27] have implicated TNF-α along with the other peptide hormones as one of those responsible for the mediation of the mammalian response to bacterial endotoxin. Considerable evidence also exists that TNF-α may play a role in the tumoricidal activity of activated macrophages [28]. Recently, human TNF-α was purified, sequenced, and cloned by recombinant DNA techniques [29]. Purified human recombinant TNF-α is a single-chain, non-glycosylated polypeptide with a molecular weight of 17.1 kDa [30]. *In vitro*, TNF-α is cytostatic or cytotoxic to a number of mammalian and human tumor cells [31]. Tusujimoto et al. [32] and Baglioni et al. [33] demonstrated that human cells in culture express high affinity (kDa of 10^{-9} to 10^{-10}) binding sites for TNF-α. Cells have been shown to contain between 100 and 5,000 receptors per cell; there has been, however, no apparent correlation between the number (or affinity) of receptors per cell and the cellular response to TNF-α antiproliferative effects [34], suggesting a mechanism beyond receptor signaling that accounts for TNF-α action.

Recent clinical studies with TNF-α were disappointing [35–38] due to the inability to achieve tumoricidal levels of TNF-α in serum and the restricted cytotoxicity of this molecule. Antibody conju-

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[c]*Abbreviations used:* CEA, carcinoembryonic antigen; TNF-α, tumor necrosis factor; LT, lymphotoxin; IFN-α, α-interferon; SPDP, *N*-succimindyl 3-(2-pyridyldithio) proprionate; ABTS, 2,2'-azino-bis (3-ethyl benzthia zoline-6 sulfonic acid); EDTA, ethylenediamine tetraacetic acid; DMF, dimethyl formamide; MEM, minimum essential medium; FBS, fetal bovine serum; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate buffered saline; Buffer A, 100 mM sodium phosphate, pH 7.0, and 0.5 mM EDTA; TEA-HCl, triethanolamine hydrocholoride; Buffer B, 5 mM bis-Tris acetate buffer, pH 5.8, 50 mM NaCl, and 1 mM EDTA; SDS-PAGE, sodium dodecyl sulfate polyacrylimide gel electrophoresis; ZME-TNF conjugate, conjugate of murine antibody ZME-018 and TNF-α; BSA, bovine serum albumin.

gates have the ability to modify the natural pharmacology and therapeutic efficacy of proteinaceous biological-response modifiers, such as IFN-α [39] and TNF-α. Accordingly, we have coupled recombinant human TNF-α to the murine antimelanoma monoclonal antibody ZME-018 using the heterobifunctional crosslinking reagent SPDP as a model system to evaluate the utility of antibody-guided delivery of endogenous cytotoxic cytokines.

MATERIALS AND METHODS

Materials. The reagents 2-iminothiolane, ABTS, and SPDP were obtained from the Sigma Chemical Co. (St. Louis, MO). EDTA (disodium salt) was purchased from Boehringer Manneheim Corp. (Indianapolis, IN). TEA-HCl was obtained from the Kodak Chemical Co. (Rochester, NY) and DMF was purchased from the Aldrich Chemical Co. (Milwaukee, WI). Tris buffer was obtained from Bio-Rad Laboratories, Inc. (Richmond, CA). Recombinant human tumor necrosis factor (0.5 mg/mL, sp. act. 3×10^7 units/mL) was obtained from Genentech Inc. (San Francisco, CA). Antibody ZME-018 was a gift from Hybritech, Inc. (San Diego, CA). Dulbecco's modified Eagle's medium was obtained from Cellgro, Inc. (Washington, D.C.). MEM was obtained from GIBCO Laboratories (Grand Island, NY). FBS was purchased from Hyclone, Inc. (Logan, UT). Horseradish-peroxidase conjugated goat antimouse antibody for ELISA was purchased from Bio-Rad Laboratories.

Cells. Human melanoma cells A375-M and AAB-527 were obtained from Dr. I. J. Fidler of the M. D. Anderson Cancer Center (Houston, TX) and Dr. Pat Trown of Xoma Corp. (Davis, CA), respectively. The A375-M cells were routinely grown at a density of 7×10^6 cells per T-75 flask in Dulbecco's MEM 10% FBS that contained gentamicin (0.05 mg/mL), added sodium pyruvate (100 mM), non-essential amino acids (10 mM), glutamine (200 mM), and MEM vitamins. The cells were routinely subcultured twice each week. The AAB-527 cells were grown in Dulbecco's MEM 10% FBS that contained gentamicin (0.05 mg/mL) and added sodium pyruvate (100 mM). Murine L-929 cells were purchased from the American Type Culture Collection (Rockville, MD) and were grown in Dulbecco's MEM 10% FBS that contained gentamicin (0.05 mg/mL) and glutamine (200 mM).

All cells were routinely tested and found to be free of *Mycoplasma* contamination using the Gen-Probe assay kit (Fisher Scientific, Houston, TX).

Conjugation of ZME-018 with TNF-α. ZME-018 (2 mL, 4.85 mg/mL) in PBS was placed in a 12 × 75 mm glass tube; a solution of SPDP (6 mg/mL) in dry DMF was added, while being vortexed with a 5-fold molar excess of SPDP. The mixture was incubated for 30 min at room temperature, and excess unreacted SPDP was removed by gel filtration on a Sephadex G-25 column (2 × 30 cm) equilibrated with Buffer A. The void-volume peaks, which contained protein, were pooled and kept on ice.

TNF-α (5 mg, 0.5 mg/mL) was added to TEA-HCl and EDTA to a final concentration of 60 mM TEA-HCl, and 1 mM EDTA, pH 8.0; 2-iminothiolane was added to a final concentration of 5 mM. The mixture was incubated for 90 min at 4°C under a stream of N_2. Excess iminothiolane was removed by gel filtration on a Bio-Rad P-6 column (2 × 40 cm) pre-equilibrated with Buffer B. The modified TNF-α that eluted in the void volumes were pooled and kept at 4°C.

Modified ZME-018 in Buffer A was mixed with modified TNF-α in Buffer B in a molar ratio of 1:10. The pH of the mixture was adjusted to 7.0 by the addition of 0.5 M TEA-HCl, pH 8.0, and the mixture was incubated for 20 hr at 4°C. The reaction mixture was purified by chromatography on Sephacryl S-300HR. High molecular weight fractions were pooled and further purified by chromatography on an affinity column.

The affinity support for TNF-α was prepared by first dialyzing 300 μg of anti-TNF-α murine antibody (TNF-β, obtained from Genentech Inc.) in 0.1 M $NaHCO_3$ buffer, pH 8.5. Affigel-10 resin (1 mL) from Bio-Rad Laboratories was transferred to a 5 mL centrifuge tube and washed three times with cold (4°C) deionized water. The antibody and the resin were admixed and gently agitated at 4°C for 24 hr. The resin was washed, and the remaining active ester groups were blocked by the addition of 1 mL of 0.1 M $NaHCO_3$, pH 8.0, and 0.1 mL of 1 M ethanolamine.

The high molecular weight fractions from S-300 chromatography (fractions 35–55) were pooled and applied to an anti-TNF-α affigel column (1 cm × 5 cm) equilibrated with PBS. The sample was loaded and washed with 50 mL of PBS to elute free antibody. TNF-α, conjugated to ZME-018, was eluted by the addition of 50 mM sodium citrate buffer, pH 3.0, which contained 150 mM NaCl. The column effluent was fractionated using a Gilson fraction collector (model FC- 80). Protein analysis of the various fractions was performed using a Bio-Rad protein assay. Fractions from the various steps were analyzed by silver-stained SDS-PAGE using a 5–15% acrylamide gradient gel.

ELISA of ZME-018 and ZME-TNF conjugate. An ELISA was performed to determine the immunoreactivity of the ZME-TNF conjugate compared to ZME-018 alone. Briefly, 5×10^4 melanoma cells (A375-M) per well were added to 96-well polyvinyl microtiter plates (Falcon Plastics, Inc., Oxnard, CA). The plates were dried for 18 hr at 37°C and then washed twice with 10 mM PBS, pH 7.4, that contained 0.1% Tween-20 and 0.02% Thimersol (washing buffer). Antibody ZME-018 or the ZME-TNF conjugate was diluted in washing buffer that contained 0.1% BSA, and then added to each well. The plates were incubated for 1 hr at room temperature. After three washes with washing buffer, 50 μL of a 1:1000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG (Bio-Rad Laboratories) was added to each well,

which were incubated for 1 hr at room temperature. After another three washes, 100 μL of substrate (1 mM ABTS), which contained 1 μL/mL of 3% hydrogen peroxide was added to each well. The reaction was stopped after 10–20 min by the addition of 5% SDS in PBS; absorbance at 405 nm was measured with an ELISA reader.

In vitro cytotoxicity of TNF-α and ZME-TNF conjugate. To examine the cytotoxicities of ZME-TNF conjugate and TNF-α, antigen-positive human melanoma cells (A375-M or AAB-527) in MEM with 10% FBS were plated on 96-well plates at a density of 5×10^3 cells per well and were allowed to adhere for 24 hr at 37°C in 5% CO_2. After 24 hr, the medium was replaced with medium that contained various concentrations of either TNF-α or the ZME-TNF conjugate.

Measurements of the effects of TNF-α and ZME-TNF conjugate on cell growth. The effects on the growth of tumor cells in culture were determined by crystal violet staining. After additional incubation for 72 hr at 37°C, the medium was aspirated, and the cell monolayers were rinsed three times with Ca^{2+}- and Mg^{2+}- free PBS. Following the final rinse, wells were tapped dry, and cells were fixed and stained by the addition of 0.5% crystal violet in 20% methanol (0.1 mL/well). Following a 10 min incubation, plates were rinsed three times in deionized water, and crystal violet was extracted from adherent cells by the addition of 0.2 mL of Sorenson's buffer per well (0.1 M sodium citrate, pH 4.2, in 50% ethanol). Cell plates were vortexed for 30 min at room temperature, and the absorbance was read at 540 nm (Model EL-309, Bio-Tek Instruments, Winooski, VT) and compared with control wells (medium alone). All values shown are means of duplicate experiments performed in octuplicate.

RESULTS

Conjugation of ZME-018 with TNF-α. Once the coupling procedure was completed, the ZME-TNF conjugate reaction mixture was applied to an S-300 gel permeation column. As shown in Figure 1A,

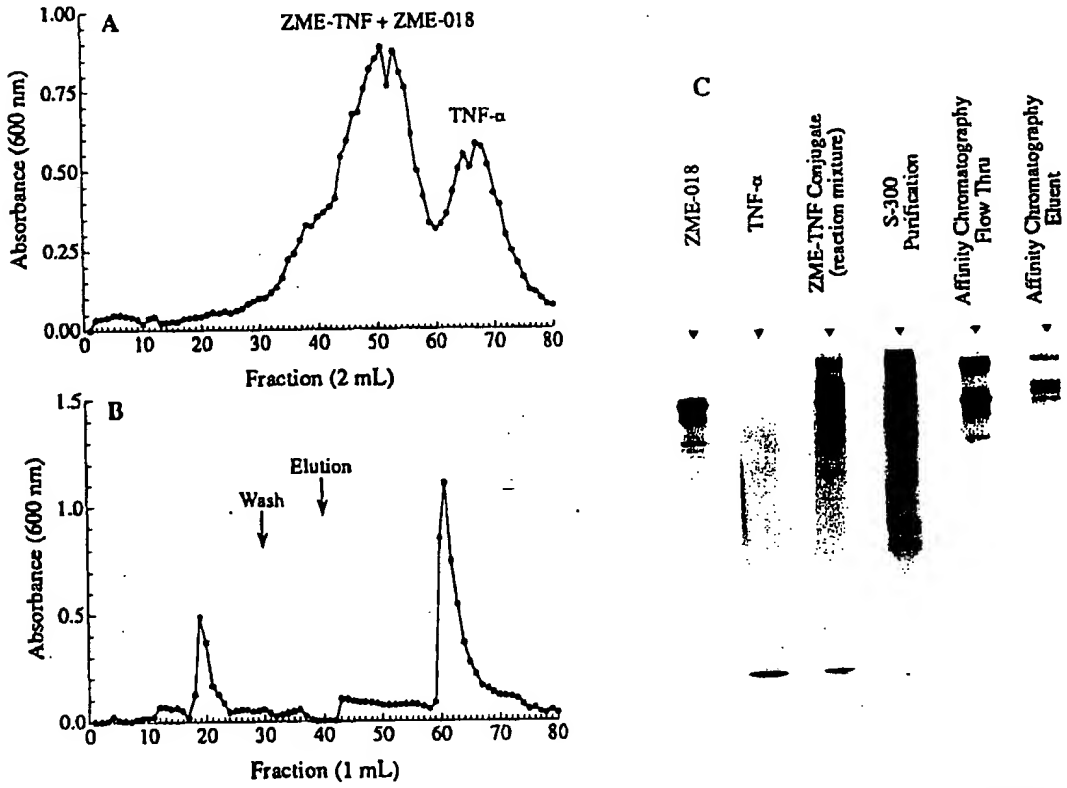

Figure 1. Analysis of the ZME-TNF conjugate reaction mixture. (A) The reaction mixture was first purified by chromatography on Sephacryl S-300HR. The two major peaks composed of ZME-018 and ZME-TNF conjugate (fractions 35–55) and free TNF-α (fractions 60–75) were separated. (B) The S-300 high molecular-weight peaks (fractions 35–55) were pooled and applied to an Affigel 10 column coupled to an anti-TNF-α antibody and equilibrated with PBS. The sample was loaded and washed with PBS to elute free ZME-018. TNF-α conjugated to ZME-018 was eluted by the addition of 50 mM sodium citrate buffer that contained 150 mM NaCl, pH 3.0. Protein analysis for collected fractions exhibited a flow-through peak (fractions 16–25) and a bound peak (fractions 59–70). (C) Fractions from the various purification steps were analyzed by silver-stained SDS-PAGE using a 5–15% acrylamide gradient gel. The ZME-TNF conjugate reaction mixture (lane 3) shows the presence of free ZME-018 and unreacted TNF-α. The high molecular-weight fraction from S-300 chromatography demonstrates the presence of free ZME-018 antibody and high molecular-weight conjugates essentially free of unreacted TNF-α. The major peak from the affinity chromatography flow-through (lane 5) contained free ZME-018 antibody and high molecular-weight component while the bound material (lane 6) was composed primarily of ZME-018 conjugated to one or two TNF-α molecules, with lesser amounts of free ZME-018.

analysis of the column effluent revealed two major peaks: a high molecular weight peak composed of free ZME-018 antibody and the ZME-TNF conjugate (fractions 35–55) and a lower molecular weight peak (fractions 60–75) composed of free TNF-α. Analysis of the pooled high molecular weight peaks of SDS-PAGE (Figure 1C) demonstrated the presence of free ZME-018 antibody and of a high molecular weight conjugate essentially free of TNF-α. Application of the high molecular weight conjugates to an anti-TNF-α affinity column (Figure 1B) demonstrated a flow-through peak that was composed primarily of ZME-018 antibody and a high molecular weight conjugate (Figure 1C).

After purification using affinity chromatography, the final product was found by SDS-PAGE to be composed of ZME-018 coupled to one TNF-α molecule (major), ZME-018 bound to two TNF-α molecules (minor), with lesser amounts of free ZME-018 (Figure 1C, lane 6). There was no detectable free TNF-α in the final preparation. The final product, however, as shown in Figure 1C also appeared to contain small amounts of a high molecular weight material. This material may have represented a small amount of antibody-TNF-α aggregates.

To determine whether the functionality of the ZME-018 antibody was compromised by covalent coupling to TNF-α, the binding of native ZME-018 and ZME-TNF conjugate to antigen-positive melanoma (A325-M) cells was assessed by ELISA. As shown in Figure 2, both ZME-018 and the ZME-TNF conjugate bound to target cells to the same extent. Neither ZME-018 nor ZME-TNF conjugate bound detectably to non-target human bladder carcinoma (T-24) cells (data not shown).

To determine the functionality of the TNF-α component of the ZME-TNF conjugate, a TNF-α bioassay was performed as previously described [25]. One unit of activity is defined as the amount of protein which will cause 50% cytotoxicity to murine L-929 cells. The activity of the final preparation was 0.6×10^6 units/mg protein. This activity translated into a final activity of 0.11×10^{15} units/mol of ZME-TNF conjugates (assuming an average molecular weight of 180 kDa). Thus, the biological activity of the ZME-TNF conjugate against L-929 cells was approximately 10-fold less than that of native recombinant TNF-α (10^{15} units/mol). Preliminary studies prior to conjugation demonstrated that chemical modification of TNF-α with the 2-iminothiolane reagent reduced the biological activity of the molecule 2-fold. Thus the reduction in biological activity of the ZME-TNF conjugate may have been due to steric factors that accompany proximity to the large immunoglobulin molecules. The cytotoxicity of the ZME-TNF conjugate was assayed against log-phase antigen-positive human melanoma (A375-M) cells in culture. A 50% growth inhibitory effect was obtained with a concentration of 30 units/mL of TNF-α alone (Figure 3). In contrast, a 10-fold lower concentration (3 units/mL) of ZME-TNF conjugate was required to achieve the same effect. Thus, against antigen-positive cells, ZME-TNF conjugate was 10-fold more active on a unit basis than native TNF-α. On a molar basis, however, the IC_{50} for TNF-α of 30 units/mL translates into a 30×10^{-12} M concentration, and the IC_{50} for ZME-TNF conjugate of 3 units/mL translates into a concentration of 27×10^{-12} M.

Maximal binding of ZME-018 antibody to target cells occurred within 2 hr of exposure at 37°C (data not shown). Continuous exposure, however, of cells to TNF-α itself for 12–18 hr was required to attain an optimal cytotoxic effect. If ZME-018 antibody binding to melanoma cells mediates the cytotoxicity of the ZME-TNF conjugate, cytotoxicity of ZME-TNF conjugate should occur with minimal exposure. A 2 hr pulse exposure of ZME-TNF conjugate or TNF-α to A375-M melanoma cells (Figure 4) demonstrated no appreciable cytotoxicity (~10%) of TNF-α under these conditions at concentrations up to 2,000 units/

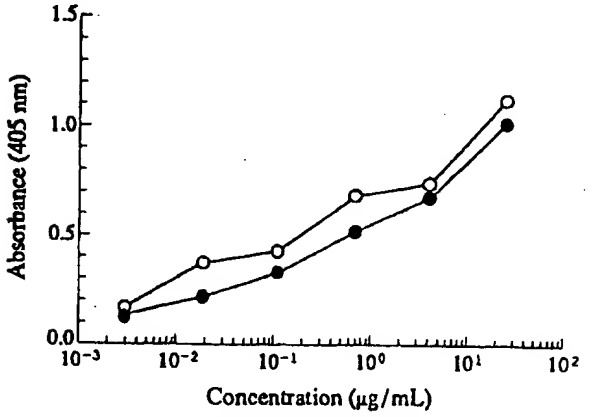

Figure 2. Determination by ELISA of the immunoreactivity of the ZME-TNF conjugate compared with ZME-018 alone in antigen positive melanoma (A325-M) cells. Both ZME-018 and ZME-TNF conjugate bound to antigen-positive A375-M melanoma cells. Key: ZME-018 (○), ZME-TNF conjugate (●).

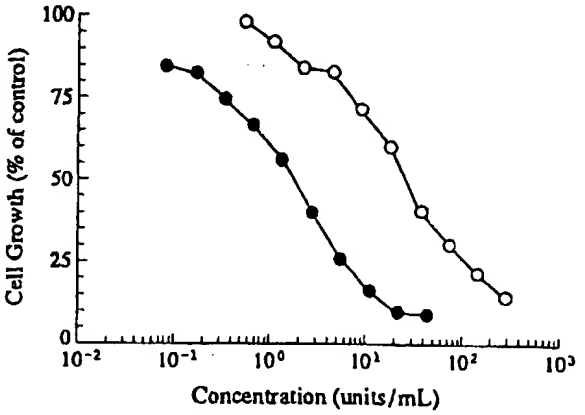

Figure 3. Cytotoxicity of ZME-TNF conjugate and TNF-α against antigen-positive human melanoma cells (A375-M). The cells were incubated for 72 hr and analyzed for relative cell proliferation by crystal violet staining. Values shown are the means of eight duplicate experiments. Key: ZME-TNF conjugate (●), TNF-α (○).

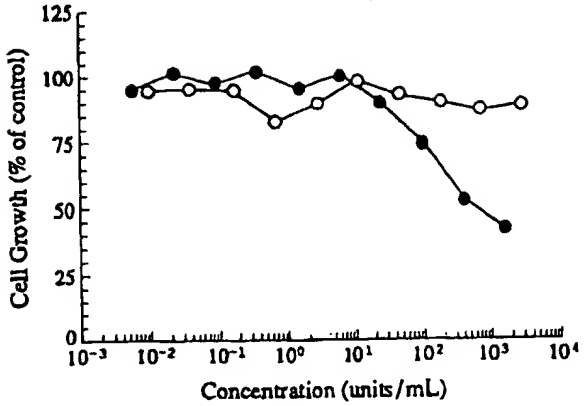

Figure 4. Pulse-exposure of TNF-α or ZME-TNF conjugate against A375-M cells. The cytotoxicity of the ZME-TNF conjugate and TNF-α alone was evaluated against log-phase human melanoma (A375-M) cells treated for 2 hr with either agent. This experiment was performed as described in Figure 3 except that 2 hr after treatment with TNF-α or ZME-TNF conjugate, the medium was removed, and the cells were washed two times with fresh medium and incubated for an additional 70 hr. Cytotoxicity was evaluated using the crystal violet assay. Key: TNF-α (○), ZME-TNF conjugate (●).

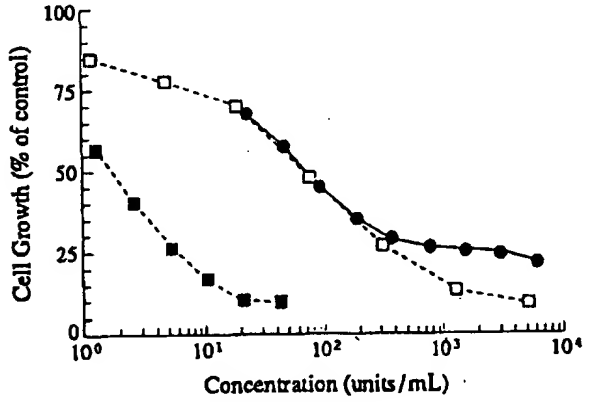

Figure 5. Effect of ZME-018 addition on the cytotoxicity of ZME-TNF conjugate. A continuous-exposure cytotoxicity assay using various doses of TNF-α or ZME-TNF conjugate was performed as described in Figure 3. Key: ZME-TNF conjugate alone (■), TNF-α alone (□), ZME-TNF conjugate + 50 μg/mL of ZME-018 (●).

mL. In contrast, ZME-TNF conjugate demonstrated 60% cytotoxicity after a 2 hr exposure of the highest dose (2,000 units/mL) tested. These studies demonstrate that the ZME-TNF conjugate required a shorter cellular exposure duration than TNF-α alone, most probably because of antibody-mediated interaction of TNF-α with either intracellular or cell-surface components.

To further examine the antibody-mediated cytotoxicity of the ZME-TNF conjugate, concentration-response curves for ZME-TNF conjugate and TNF-α were generated against antigen-positive human melanoma cells in the presence or absence of either ZME-018 or an irrelevant, murine antibody (Figure 5). In the presence of irrelevant antibody, the ZME-

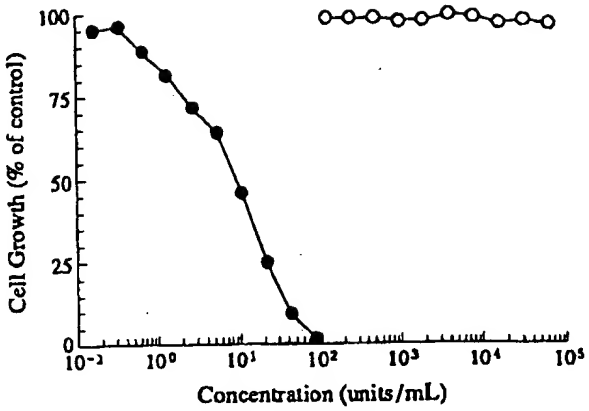

Figure 6. The cytotoxicity of ZME-TNF conjugate evaluated against an antigen-positive, TNF-α-resistant, human melanoma cell line (AAB-527). A 72 hr continuous-exposure cytotoxicity assay was performed as described in Figure 3 using log-phase AAB-527 cells. Key: ZME-TNF conjugate (●), TNF-α (○).

TNF conjugate exhibited 50% cytotoxicity (IC_{50}) at a concentration of 2 units/mL. In the presence of free ZME-018 (50 μg/mL), the cytotoxicity of the ZME-TNF conjugate was suppressed and generated a concentration-response curve similar to that of TNF-α alone. Thus, the augmented cytotoxicity of the ZME-TNF conjugate compared with TNF-α alone appeared to be mediated by specific binding to the cell surface antigen recognized by the ZME-018 antibody.

The cytotoxic effects of the ZME-TNF conjugate were tested against various cell types. Against TNF-α-resistant antigen-negative bladder carcinoma (T-24) cells, the ZME-TNF conjugate did not demonstrate growth-modulating effects (cytotoxic or growth-stimulatory). However, against antigen-positive TNF-α-resistant human melanoma (AAB-527) cells (Figure 6), the ZME-TNF conjugate demonstrated 50% cytotoxicity at a concentration of 10 units/mL. At a concentration of 100 units/mL, the ZME-TNF conjugate showed more than 95% growth inhibition against target cells. In contrast, TNF-α alone at concentrations of up to 100,000 units/mL showed no cytotoxic effects. Therefore, the ZME-TNF conjugate demonstrated impressive growth inhibition effects against cells resistant to TNF-α alone. Since this effect was observed only with antigen-positive cells, the data support the hypothesis that the observed increase in cytotoxicity was mediated by the binding of ZME-018 antibody to the cell surface.

DISCUSSION

The ability of a murine antibody to augment the cytotoxic properties of TNF-α action has several possible explanations. The antibody may hold TNF-α at the cell membrane in proximity to the TNF-α receptor and, thus, result in improved recognition of the TNF-α ligand by the TNF-α receptor. Alternatively, the interaction of the ZME-TNF conjugate with the TNF-α receptor may differ from that of TNF-α alone and may modulate or interfere with the nominal cel-

lular processing of the TNF-α receptor-ligand complex [40]. On the other hand, internalization of active TNF-α via the ZME-TNF conjugate complex may allow interaction of TNF-α ligand with intracellular receptors for TNF-α and may generate an intracellular cytotoxic signal.

Our group [41] and others [42, 43] have shown that IFN-α can also be successfully coupled to murine monoclonal antibodies to create an antiviral and cytotoxic agent that is more selective and effective than free IFN-α. Since biological response modifiers such as TNF-α and IFN-α may suffer from poor tumor selectivity and a short biological half-life, coupling to monoclonal antibodies may confer selectivity and augmented sensitivity to otherwise non-specific cytotoxic agents.

If *in vivo* targeting studies demonstrate localization of the ZME-TNF conjugate within tumors, this reagent may be clinically useful for tumor-targeted delivery of the biological response modifier TNF-α. In addition, antibody-mediated delivery of biological response modifiers may, in general, provide a new generation of tumor-directed agents with improved selectivity and greater biological activity.

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APPENDIX 3

RELATED PROCEEDINGS APPENDIX

NONE

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